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Immunohistochemical localization of chromogranin A in gonadotrophs and somatotrophs of the turkey and chicken pituitary

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Abstract

In the course of producing monoclonal antibodies to turkey prolactin, three monoclonal antibodies to turkey chromogranin A (CgA) were also produced, apparently arising from minor contamination of the turkey prolactin immunogen with peptide fragments of CgA. The identity of the antigen recognized by these antibodies was established by tandem mass spectrometry de novo sequencing of seven tryptic peptides from a turkey pituitary protein purified by immunoaffinity chromatography. These peptides showed high homology with distinctly separate regions of mammalian and ostrich CgA, and in silico cloned chicken CgA sequences. Chromogranin A immunostaining patterns on Western blots and pituitary tissue sections differed from those of prolactin, growth hormone, or luteinizing hormone (LH). Dual-label fluorescent immunohistochemistry revealed that CgA was co-localized with LH in most avian gonadotrophs in young chickens and turkeys, but not in adult, laying birds. Conversely, CgA was found in a majority of somatotrophs in laying birds but was absent from somatotrophs in young, growing chickens and turkeys. Lactotrophs contained no detectable CgA immunoreactivity in the tissues studied. These results suggest that CgA may modulate hormone secretion by gonadotrophs and somatotrophs in a manner that differs between cell type with age or reproductive state.

Keywords: Turkey; Chicken; Chromogranin A; Amino acid sequence; Somatotroph; Gonadotroph

1. Introduction

Chromogranin A (CgA) is a secretory protein of the "granin" family, which includes CgA, chromogranin B (CgB/secretogranin I), and chromogranin C (CgC/secretogranin II). These granins are found in the secretory granules of endocrine and neuroendocrine cells, and are released from the cell along with the hormonal component of the granule in response to a stimulus. The formation of secretory granules at the trans-Golgi network is essential for sorting of hormones into the regulated secretory pathway and for processing of prohormones to the appropriate secretory product. Recently, Kim et al. (2001) have shown that dense-core secretory granule formation can be controlled by CgA. In their study, the presence of CgA was sufficient to turn on secretory granule formation in endocrine cells and, therefore, regulated secretion of that cell's hormone(s). These authors demonstrated that cells lacking CgA exhibited only constitutive secretion of hormone and did not respond to regulatory stimuli, and that initiation of CgA synthesis within the cell was sufficient to establish regulated secretion of the hormone. Several studies have reported localization of CgA in gonadotrophs of the rat (Montero-Hadjadje et al., 2002; Watanabe et al., 1991, 1998a,b) and bovine (Bassetti et al., 1990) anterior pituitary and in gonadotrophs of the chicken pars tuberalis (Kameda et al., 1998). A putative CgA peptide has been isolated from the ostrich pituitary (Lazure et al., 1990).

The present study was undertaken to produce monoclonal antibodies to turkey prolactin (PRL). While

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screening our antibody-producing hybridomas by immunohistochemistry (IHC) on sagittal sections of turkey and chicken pituitary, we encountered several monoclonal antibodies yielding a staining pattern that did not correspond to any of the characteristic avian pituitary cell types. Dual-labeling studies showed that these monoclonal antibodies did not bind to lactotrophs, but did bind to some gonadotrophs and/or somatotrophs depending on the age/reproductive state of the animal. By use of immunoaffinity chromatography and tandem mass spectrometry (MS) de novo sequencing of tryptic peptides, the antigen protein was identified as CgA. Accordingly, we here report on the production and characterization of monoclonal antibodies to turkey CgA, and describe marked differences in IHC staining of CgA in gonadotrophs and somatotrophs of immature and adult turkeys and chickens.

2. Methods

2.1. Monoclonal antibody production

The procedures for monoclonal antibody production and screening have been described previously (Berghman et al., 1988; Proudman et al., 1999). Briefly, mice were immunized subcutaneously four times with 25 μg of purified turkey PRL (Proudman and Corcoran, 1981) at 3 week intervals. This preparation contained a mixture of non-glycosylated and glycosylated isoforms of monomeric PRL. Mice were boosted by intraperitoneal injection of 50 μg of turkey PRL 4 and 2 days prior to fusion. All mice were maintained under standard methods for humane treatment established by the Katholieke Universiteit Leuven and sacrificed by cervical dislocation. A suspension of activated splenocytes was prepared, fused with SP2/0 myeloma cells by electrofusion, and plated into 96-well plates at a density of 20,000 cells/well. Plates were screened two weeks after fusion using an IHC screening protocol (Berghman et al., 1989). Pituitaries from laying turkey hens and juvenile broiler chickens were fixed by immersion overnight in Bouin-Hollande Sublimate (see Proudman et al., 1999) and stored in 50% ethanol until processing. Tissues were processed through graded concentrations of alcohol followed by Histo-Clear and paraffin infiltration using a Tissue-Tek 1000 Vacuum Infiltration Processor (Spectron, Kirkland, WA 98033). Culture media from microtiter plate wells containing hybridomas were incubated overnight on sagittal 5 μm pituitary sections and antibody binding was detected using peroxidase-labeled goat anti-mouse IgG (Dako, Heverlee, Belgium) followed by chromogenic detection using diaminobenzidine. Positive cultures producing relevant IHC staining patterns were propagated and cloned by limiting dilution.

2.2. Immunohistochemical characterization of monoclonal antibodies

The monoclonal antibodies were initially characterized using the IHC screening protocol described above. Final characterization of pituitary cells containing the antigen employed 4–6 μm sagittal sections of pituitaries from 5-week-old turkey poults, laying turkey hens between 32 and 55 weeks of age, 3-week-old broiler chickens and laying Leghorn hens at the end of the reproductive season (>70 weeks of age). All animal procedures were performed in accordance with protocols approved by the Beltsville Area Institutional Animal Care and Use Committee. Lactotrophs expressing turkey or chicken PRL were detected using a rabbit antibody to recombinant chicken PRL diluted 1:4000 (Lopez et al., 1995). Somatotrophs producing turkey or chicken growth hormone (GH) were detected using a rabbit antibody to affinity-purified chicken GH diluted 1:20,000 (Berghman et al., 1988). Gonadotrophs containing turkey or chicken luteinizing hormone (LH) were detected using a rabbit anti-chicken LH serum diluted 1:10,000 (USDA-AcLH-5; Proudman et al., 1999). Immunostaining with CgA monoclonal antibodies was accomplished by diluting ascites fluid between 1:10,000 and 1:30,000. Dual-label fluorescent IHC was performed as described previously (Proudman et al., 1999), except that biotinylated goat anti-rabbit IgG (1:400) was purchased from Vector Laboratories (Burlingame, CA) and Rhodamine Red-X-conjugated anti-mouse IgG (1:200; Jackson) was used instead of the Lissamine-conjugated antibody. By this procedure, cells immunoreactive to the newly produced monoclonal antibodies stained red, while those immunoreactive to the previously characterized PRL-, GH-, or LH-antibodies stained green.

2.3. Microscopy

Fluorescent images were acquired in frame mode with a Zeiss LSM 410 (Carl Zeiss, Thornwood, NY) confocal microscope through a 63× C-apochromat 1.2 NA water immersion objective or a 25× plan neofluor 0.8 NA multi-immersion objective. The 488 and 568 nm lines of an Omnichrome (Omnichrome, Chino CA) Ar/Kr laser were used to excite green and red emitting fluorochromes, respectively. A band-pass 515–540 nm filter was used for green emitting fluorochromes, and long-pass 590 nm filter for red emitting fluorochromes. Individual optical sections were digitally recombined into a single composite image using LSM software (Carl Zeiss, Thornwood, NY). Brightfield images were acquired with a Zeiss Axioscope (Carl Zeiss) fitted with a Spot (Diagnostic Instruments, Sterling Heights, MI) digital camera.

2.4. Antibody specificity

Potential cross-reactivity of our monoclonal antibodies with turkey PRL, chicken GH, and chicken LH was assessed by analysis of the banding patterns of purified hormones and pituitary extracts on Western blots of denaturing SDS–PAGE gels and of isoelectric focusing (IEF) gels. Pre-cast gels (10% NuPage Bis-Tris SDS gels; pH 3–10, IEF gels) were electrophoresed and blotted according to manufacturer's instructions (Novex, San Diego, CA). The membranes (Immobilon-psq; Millipore, Bedford, MA) were exposed to primary antibody for 3 h at room temperature after blocking with 5% non-fat dried milk powder in water. Antibody solutions (1:1000 dilution) were prepared in Tris-saline buffer containing 0.1% Triton X-100, pH 7.4 (TBS) and 1% (w/v) BSA. Detection of bound antibody was accomplished by incubating membranes for 1 h at room temperature with biotinylated goat anti-rabbit IgG or biotinylated goat anti-mouse IgG (Vector Laboratories) diluted 1:1,000. After washing in TBS, the membranes were incubated with streptavidin–alkaline phosphatase (Jackson) as described for IHC. Color was developed using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate as substrate to produce a purple precipitate (Berghman et al., 1992). Molecular weights of immunostained bands on SDS blots were estimated by image analysis of scanned blots (Quantity One, PDI, Huntington Station, NY) and comparison to pre-stained standards (Multimark, Novex).

2.5. Immunoaffinity chromatography

Antigens recognized by selected monoclonal antibodies were isolated from a turkey pituitary extract by immunoaffinity chromatography (IAC). Mouse IgG was isolated from ascites fluid using immobilized Protein A (Protein A–Sepharose 4 Fast Flow, Amersham Biosciences, Piscataway, NJ) and coupled to CNBr-Activated Sepharose 4B (Amersham Biosciences). This immobilized antibody was used for affinity purification of pituitary proteins from turkey pituitaries homogenized 5:1 (w:v) in 0.05 M sodium phosphate buffer containing 0.28 M sodium chloride, 0.05 M lactose, and 1 M urea, pH 7, in the presence of a cocktail of protease and glycosidase inhibitors (10 mM 4-aminobenzamidine dihydrochloride; 0.5 μg/ml leupeptin; 0.7 μg/ml pepstatin; 0.5 mM Pefabloc SC; 2.5 mM mercuric chloride; 2 μg/ml phosphotungstic acid; 0.1 mM 4-chloromercuribenzoic acid). Homogenization of the tissue with a Brinkman Polytron (Brinkman Instruments, Westbury, NY) was followed by a freeze–thaw cycle, re-homogenization, and centrifugation (Proudman et al., 1995). Binding of pituitary proteins to immobilized antibody was accomplished batchwise by end-over-end rotation overnight at 4 °C. The slurry containing the immunoaffinity gel beads and pituitary extract was transferred to a 1.5×7.5 cm disposable column and the gel was rinsed with Pierce Gentle Binding Buffer (Pierce Chemical, Rockford, IL). Bound proteins were eluted with Pierce Gentle Elution Buffer, extensively dialyzed against 0.05 M Tris–HCl, pH 8, and then against 5 mM ammonium bicarbonate, pH 9, followed by lyophilization.

2.6. Primary structure determination

Identification of the pituitary protein purified by IAC using monoclonal antibody PR1D8E10 was initially attempted with conventional gas-phase sequencing based on Edman degradation, but the protein appeared to be N-terminally blocked. The sample was then digested by trypsin and analyzed by Q-Tof MS (Micromass UK, Manchester, UK; Morris et al., 1997). Tryptic peptides were prepared as follows: The lyophilized protein sample was first dissolved in a few microliters of double distilled water. The exact concentration could not be determined, since the protein sample appeared to contain residual salt even after extensive dialysis. One μl of this protein solution was added to a microcentrifuge tube with a solution of 50 mM ammonium bicarbonate and 5 mM calcium chloride containing 1 μg of bovine pancreas trypsin (Sequencing grade, Fluka). The trypsin digestion was allowed to proceed overnight at 37 °C. The resulting peptides were desalted using ZipTip C18 pipette tips (Millipore). Prior to use, the ZipTips were washed in 100% acetonitrile (HPLC grade, Riedel-De Haën, Seelze, Germany) and 50% acetonitrile in water. Then the tips were equilibrated in 0.1% trifluoroacetic acid. Twenty μl of 1% formic acid in water was added to the peptide solution and the resulting solution was bound to the C18 silica. Repeated washing and desalting of the peptides was done in 0.1% trifluoroacetic acid followed by 0.1% formic acid in water. The peptides were then eluted in 3 μl of 90% acetonitrile-1% formic acid in water. This solution was deposited into a glass capillary (F-type, Micromass), which was fitted in the nano-electrospray source of the Q-Tof mass spectrometer. An MS survey spectrum was acquired, after which individual peptides were selected and subjected to collision-induced dissociation, yielding MS/MS spectra of the fragmented peptides. De novo sequence information was derived from the MS/MS spectra with the aid of MaxEnt 3 software (deconvoluting and deisotoping of data) and the PepSeq tool of Micromass' Biolynx software package.

Homology database searching with the deduced amino acid sequences was done with the Blitz engine (www.ebi.ac.uk/bic_sw, currently replaced by Mpsrch tool at www.ebi.ac.uk/MPsrch), set to query the SWALL protein database using default parameters. Similarity searching with linked peptides was done using

the Fasta engine at www.ebi.ac.uk/fasta33, with the fasts3 program set to search the SWALL database.

2.6.1. In silico cloning of the chicken CgA cDNA

Expressed sequence tag (EST) database queries were run on the WU-Blast2 engine at www.ebi.ac.uk/blast2, using either the WU-tblastn program (for protein → nucleotide queries) or the WU-blastn (for nucleotide → nucleotide queries) program in combination with the Vertebrate EST database. Translated BLAST searching (protein → nucleotide) with peptide 6 (Table 1) in the vertebrate EST database produced hits in the "ESTs from Normalized Chicken Pituitary/Hypothalamus/Pineal cDNA library," with similarity to bovine chromogranin A. These sequences were extended to the 5′ end using new overlapping BLAST queries (nucleotide → nucleotide) in the vertebrate EST database, all yielding hits in the same cDNA library. Sufficient searches were conducted to ensure at least triple confirmation for each base position. This was achieved with an alignment (using the ClustalW tool at www.ebi.ac.uk/clustalw and the GeneDoc 2.6 software (Nicholas et al., 1997)) of the following 11 clones: Accession Nos. BI393286, BI394461, BI393833, BI389669, BI394522, BI392934, BI393296, BI390023, BI391666, BI393204, and BI393301. DNA to protein translation was performed with the Transeq tool at www.ebi.ac.uk/emboss/transeq. All database search engines and server queries were run with default parameters.

3. Results

Screening of fusion plates using IHC revealed numerous wells containing monoclonal antibodies that bound to cells present only in the cephalic lobe of the turkey anterior pituitary in a pattern similar to that reported previously for a monoclonal antibody to a synthetic PRL peptide (Berghman et al., 1992; Ramesh et al., 1996). In the laying bird, lactotrophs are restricted to the cephalic lobe while somatotrophs are found only in the caudal lobe (Berghman et al., 1993). One such antibody, PR1G2B6, produced robust staining of lactotrophs in both turkey and chicken pituitary sections (Figs. 1B and D, respectively) and completely co-localized by immunofluorescent staining with cells immunostained by a rabbit polyclonal antibody to recombinant chicken PRL (data not shown).

In contrast, three independently derived monoclonal cell lines (PR1D8E9, PR1D8E10, and PL1G7F11) produced a staining pattern in both lobes of the anterior pituitary, with the greatest concentration of stained cells in the caudal lobe (Figs. 1A and C). Only gonadotrophs are known to populate both lobes of the avian anterior pituitary, but the immunostaining patterns for LH and FSH are markedly different from that observed with these monoclonal antibodies (Proudman et al., 1999). The population of immunostained cells was clearly more dense in the caudal lobe than in the cephalic lobe, while gonadotrophs are characteristically distributed evenly in both lobes. Immunostaining of sagittal sections of the frog pituitary showed a wide distribution of immunoreactive cells throughout the anterior pituitary, suggesting that the target of these antibodies may be a highly conserved protein (data not shown).

Dual-label fluorescent IHC was employed to determine if the protein immunostained with these antibodies was co-localized with PRL (the immunogen), LH (chosen because of its wide distribution in both lobes of the anterior pituitary), or GH (chosen because of its presence unique to the caudal lobe). In young chickens, co-localization was observed in most LH-immunoreactive gonadotrophs (Figs. 2A and B), but not in somatotrophs (Fig. 2C). In adult laying hens, co-localization in gonadotrophs was exceedingly rare (Fig. 2D), while co-localization was readily apparent in many, but not all, adult somatotrophs (Fig. 2E,F). Results with immature and laying turkeys were similar. The protein recognized by these monoclonal antibodies was not co-localized with PRL in any of the tissues studied (data not shown).

Table 1
Non-redundant de novo sequencing results for the peptides derived from the IAC-purified protein. Leu/Ile ambiguity is always shown as Leu (L)

Peptide no.	Observed m/z	Charge state	Sequence
1	672.29	2+	YDFSSPEEDVR[a]
2	808.93	2+	NSLELEEEGEQPSR
3	913.47	2+	DAEDVQGEAALDNHVDK[b]
4	943.00	2+	LEDEGMQAEEDTFQSR
5	1085.00	2+	SGFEDELSEVLESQNDENK
6	1101.45	2+	RTEDQELESLAALEAELER[b,c]
7	999.60	3+	1484.58 + VEVLSDTLSKPDPL[d]

[a] This peptide was also found carrying a phosphorylated Ser residue.
[b] The triply charged counterparts of these peptides were also present.
[c] Twelve truncations and four unintelligible extensions of this peptide were also found, sometimes both as doubly and triply charged peptides.
[d] No complete sequence could be derived, likely because of a disulfide bond within the peptide. The probable full peptide (as derived from chicken CgA) is CIVEVISDTLSKPDPLPISEECLETLR with disulfide bond between the two Cys residues.

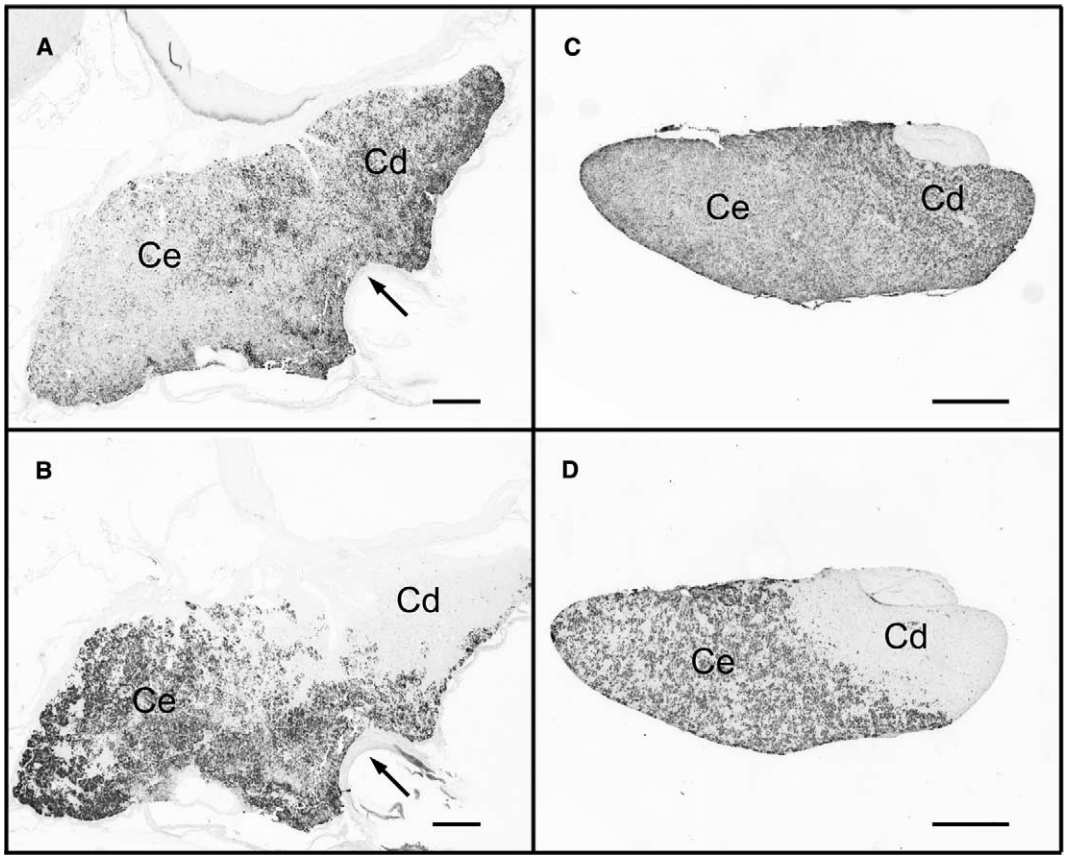

Fig. 1. Photomicrographs of sagittal pituitary sections from laying turkey (A and B) and chicken (C and D) immunostained with monoclonal antibodies to chromogranin A (PR1D8E10; A and C) or to prolactin (PR1G2B6; B and D). Lactotrophs were present primarily in the cephalic (Ce) lobe of the anterior pituitary, whereas cells containing chromogranin A were found in both cephalic and caudal (Cd) lobes. The arrow indicates the approximate anatomical demarcation of cephalic and caudal lobes in the turkey pituitary. Calibration bars: 500 μm.

Immunoaffinity chromatography was employed to isolate the target protein from turkey pituitaries. All three antibodies were evaluated, and PR1D8E10 was found to yield the highest recovery of extracted protein based on UV absorbency of eluted protein. The IAC eluate was digested with trypsin. Mass spectrometric analysis of the digest, with de novo sequencing of the peptides, revealed full or partial sequences for 26 peptides. These could be reduced to seven non-redundant sequences (Table 1). It must be noted that Leu and Ile could not be distinguished by this technique because of their identical mass, and are always indicated as Leu.

Peptides 5, 6, and 7, either separately in the Blitz or MPsrch engine, or together in a linked peptides query using the Fasta engine, showed significant similarity to CgA of various species. The four remaining peptides could only be matched to CgA following the in silico cloning of chicken CgA cDNA, because no complete avian CgA protein sequence was previously available. For this purpose, the compound consensus cDNA sequence of 11 chicken EST clones was used to derive the chicken CgA amino acid sequence. Fig. 3 shows the alignment of all seven de novo sequenced peptides from Table 1 to the derived chicken CgA protein, as well as seven complete sequences of other species and a partial ostrich sequence (Lazure et al., 1990).

As suspected, the homology between avian and mammalian CgA in the N-terminal and C-terminal portions is markedly higher than in the central part. This explains why the identification could initially only be based on peptides 5, 6, and 7 (Table 1). Apart from the Leu/Ile ambiguity, there are some minor differences between chicken CgA sequence and the turkey CgA peptides, merely representing a single base mutation (Ala–Val; Thr–Ala) or a basic cleavage site swap (Lys–Arg).

Putative turkey CgA was characterized by SDS–PAGE and IEF. Both IAC-purified CgA and CgA-immunoreactive (ir) proteins from turkey and chicken pituitary extracts showed considerable size heterogeneity (Fig. 4). Prominent bands were observed in the IAC-purified preparation at 71, 55, 22, 20, 13, 9.9, and 8.8 kDa. Quite similar bands were observed in both bird species. In addition, a prominent 9.9 kDa CgA-ir band was observed in the tPRL preparation, while less prominent bands were observed in both tPRL and pu-

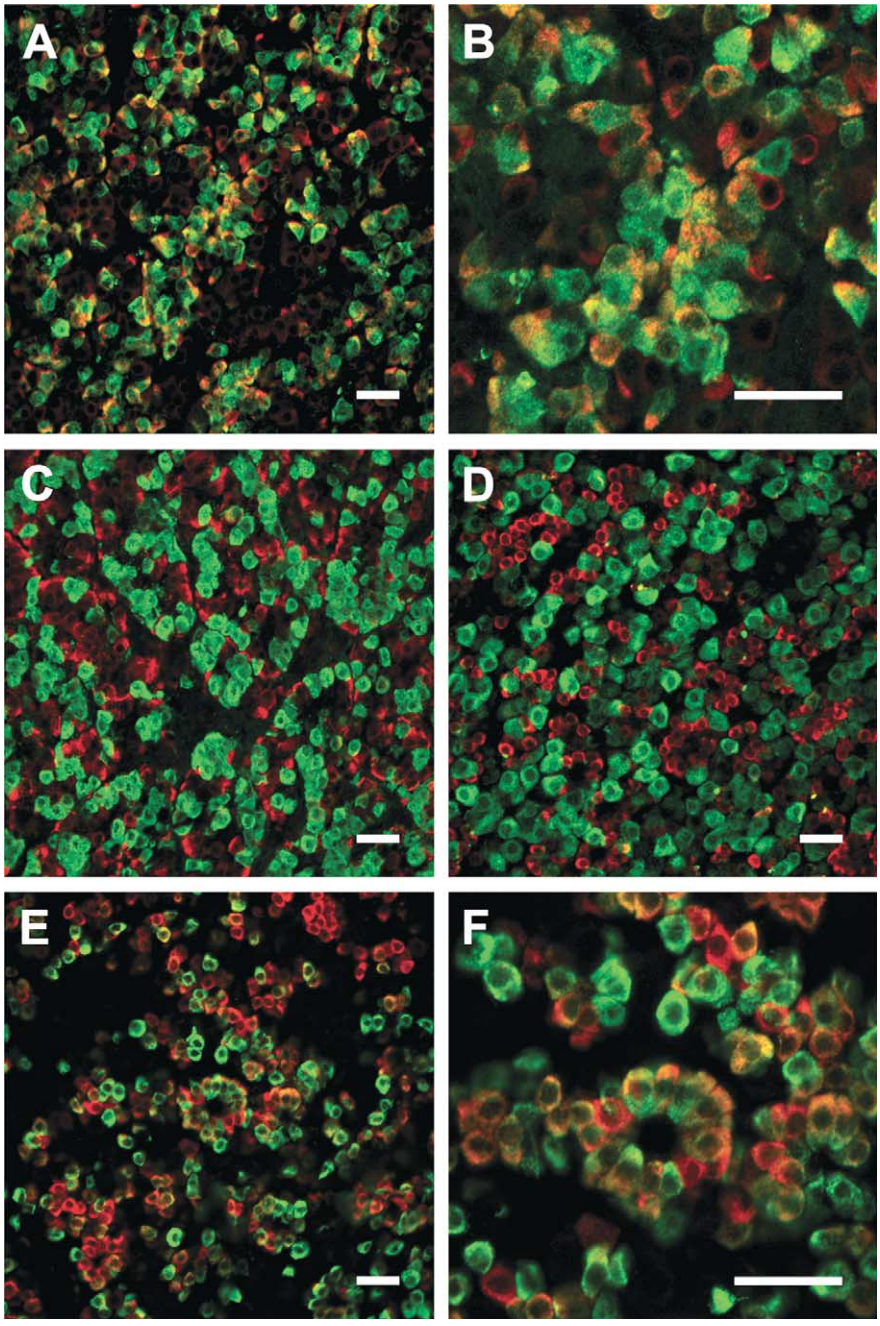

Fig. 2. Dual-label immunofluorescent staining of sagittal pituitary sections from immature (3-week-old) broiler chickens (A and B) and laying chickens (C). CgA immunoreactivity (red) was widely co-localized with LH (green) in gonadotrophs in the young chicken pituitary (A and B). In the mature laying hen, relatively little CgA immunoreactivity was found in LH-containing gonadotrophs (C). In contrast, GH-immunoreactive somatotrophs (green) were largely devoid of CgA in the young chicken (D). Somatotrophs of adult hens (E and F) ranged widely in CgA immunoreactivity, with organized groups of somatotrophs containing cells apparently devoid of CgA in close association with somatotrophs intensely immunoreactive for CgA. Calibration bars: 25 μm.

rified chicken GH (Fig. 4). No staining of purified chicken LH was observed with the CgA monoclonal antibodies (data not shown). Western blots stained with PR1G2B6 (antibody to turkey PRL) revealed characteristic staining of purified turkey PRL and of the endogenous PRL contained in pituitary extracts of chicken and turkey (Figs. 4,5). Monoclonal PR1G2B6 and polyclonal antibodies to GH or LH failed to recognize IAC-purified turkey CgA. Isoelectric focusing of IAC-purified CgA and pituitary extracts revealed remarkably little heterogeneity in the isoelectric point (pI) of CgA-ir proteins (Fig. 5). The pI was quite similar to that of turkey PRL (previously shown to be 4.56–5.44; Proudman and Corcoran, 1981) and purified tPRL showed

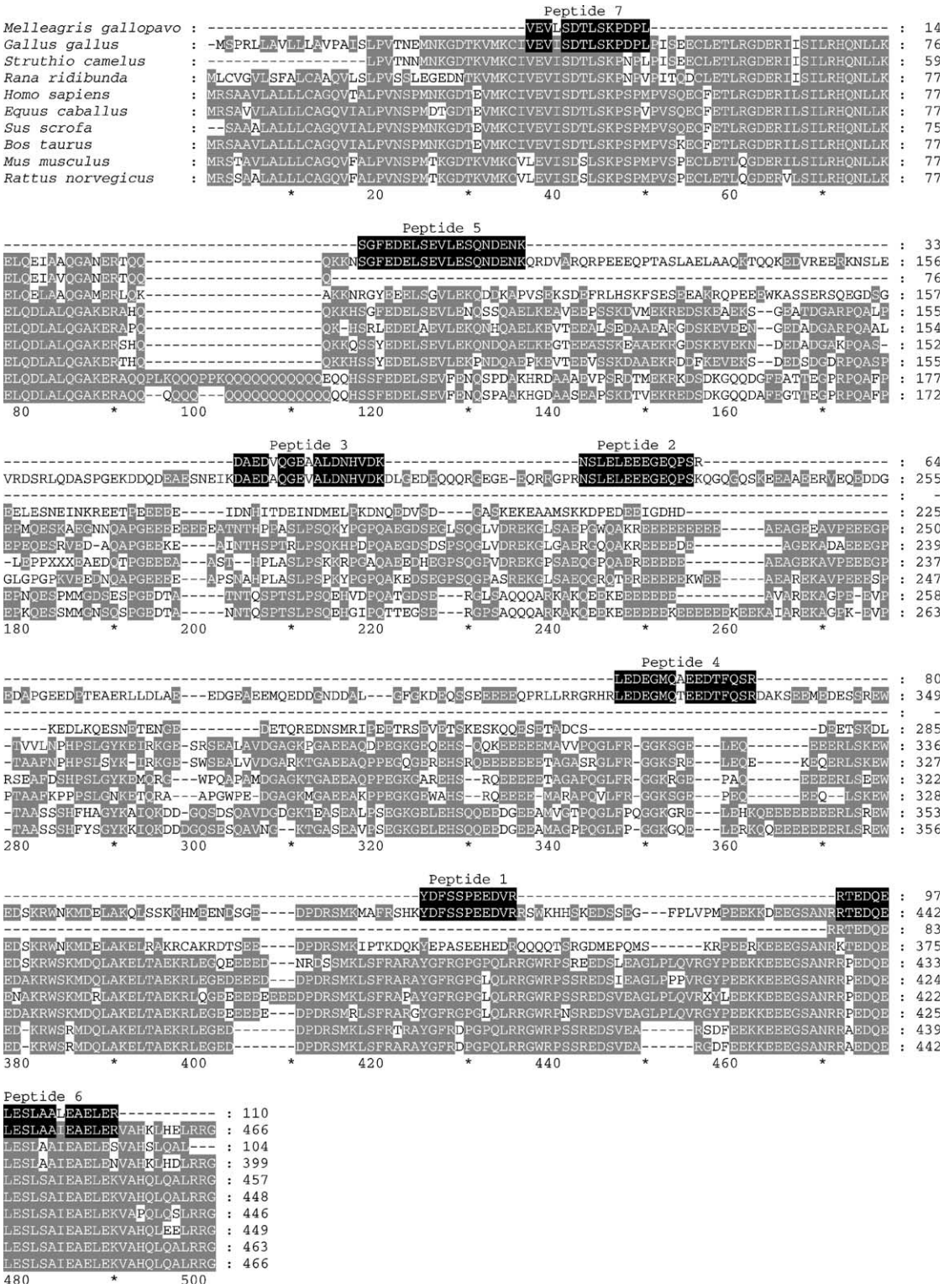

Fig. 3. Alignment of the in silico cloned chicken (*Gallus gallus*) CgA with a partial ostrich sequence and the complete CgA sequences of one amphibian and six mammalian species. Grey shades represent identical amino acids at each position. The de novo sequenced turkey (*Meleagris gallopavo*) peptides (cf. Table 1) are listed in the top line of each sequence block, with amino acids identical to the chicken sequence shaded in black.

both CgA-ir and PRL-ir. The pI of turkey GH was much higher than that of CgA (7.9–8.2; Proudman and Opel, 1990). Chicken LH, with a pI of 9.55 (Krishnan et al., 1994), remained near the cathode and was not immunostained by the monoclonal antibodies to CgA or PRL (data not shown).

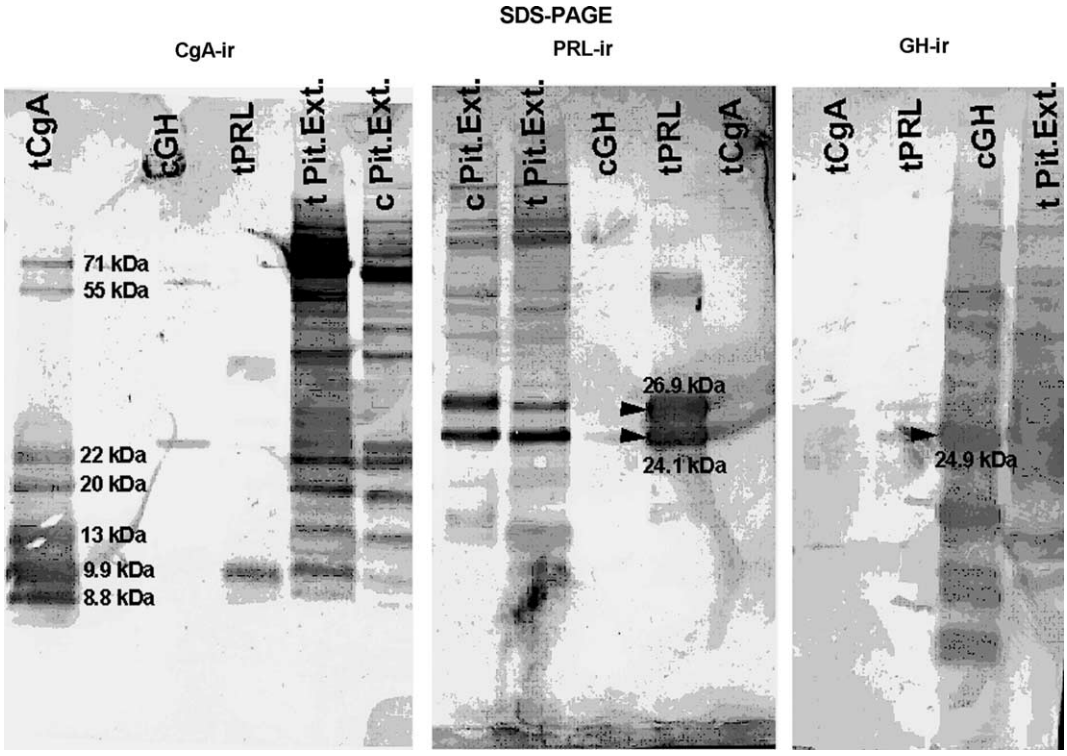

Fig. 4. Western blots of SDS–PAGE separations of IAC-purified CgA, purified turkey PRL, purified chicken GH (AFP7678b), and chicken and turkey pituitary extracts, immunostained with monoclonal antibodies to CgA (PR1D8E10), turkey PRL (PR1D2B6), or polyclonal rabbit antibody to IAC-purified chicken GH (Berghman et al., 1988). Molecular weights were estimated from standards.

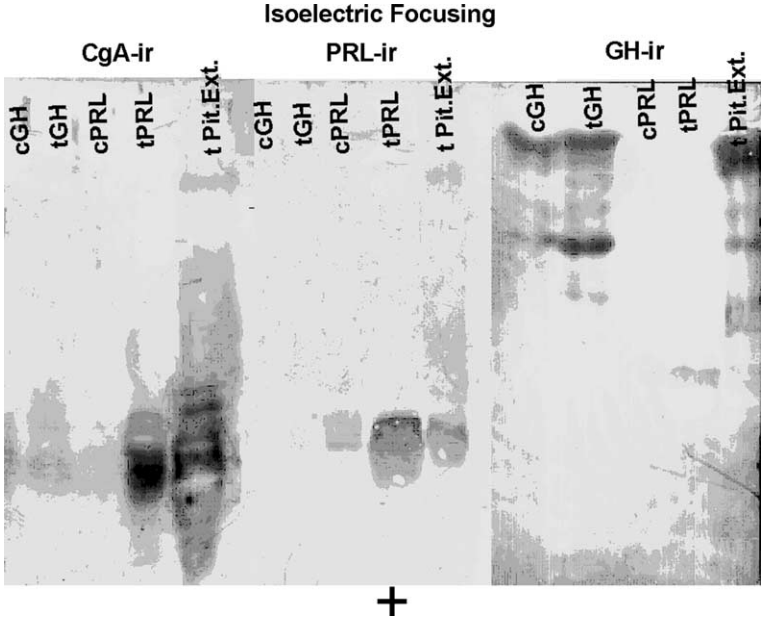

Fig. 5. Western blots of isoelectric focusing gels separating purified turkey PRL, IAC-purified chicken PRL, purified chicken GH (AFP7678b), purified turkey GH, and turkey pituitary extract, immunostained with monoclonal antibodies to CgA (PL1G7F11), turkey PRL (PR1G2B6), or polyclonal rabbit antibody to IAC-purified chicken GH (Berghman et al., 1988). Anode is designated by (+).

4. Discussion

Production of monoclonal antibodies to turkey PRL unexpectedly produced antibodies to an unrelated protein, CgA. These antibodies likely arose due to contamination of the immunogen with a 9.9 kDa peptide fragment of this protein that is of a similar pI to turkey PRL. We were able to detect these unexpected anti-

bodies because we employed a screening procedure using tissue sections rather than the more common procedures, which use binding to purified immunogen. The characteristic distribution of classical pituitary cell types in avian species allowed easy recognition of this staining pattern as distinct from that expected for the immunogen employed and, indeed, distinct from that of any pituitary cell type described so far.

The identification of the antigen as CgA was initially based on sequence homology of three de novo sequenced tryptic peptides of IAC-purified protein. Using the predicted protein sequence of the in silico cloned chicken CgA cDNA, all non-redundant de novo sequenced peptides were shown to be CgA-derived.

Although Leu and Ile can be annotated in the MS/MS derived sequences based on homology with other sequences, we preferred to annotate a Leu in all cases and to note the ambiguity. For peptide 7 (Table 1) only a partial sequence could be obtained. This is likely due to the occurrence of a conserved disulfide bond between C34 and C55 (cf. Fig. 3; Hendy et al., 1995). Indeed, the tryptic peptide CIVEVISDTLSKPDPLPISEECLETLR (predicted from chicken CgA), containing a disulfide bond, exactly accounts for the mass of peptide 7.

The observed multiple bands for CgA-ir proteins in SDS and IEF blots is consistent with the proteoglycan structure of this protein and its known role as a precursor for bioactive peptides (Eskeland et al., 1996). These authors demonstrated extensive processing of CgA on SDS–PAGE immunoblots from various mouse tissues. Intact CgA showed a MW of ~80 kDa, but higher mass CgA-ir bands were observed that were consistent with the presence of proteoglycans and many lower mass bands were attributed to proteolytic processing. Kameda et al. (1998) similarly observed multiple CgA bands on immunoblots of chicken pars distalis and pars tuberalis, with the prominent band having a mass of 75 kDa. This is similar to the mass of the most prominent band observed in our immunoblots of chicken and turkey pituitary extracts (Fig. 4). Our results demonstrating that the amino acid sequence of proteins purified from turkey pituitaries by IAC could all be mapped to the in silico cloned chicken CgA sequence provide confidence that the monoclonal antibody does not recognize other pituitary proteins.

The observed age-related changes in the presence of putative CgA in pituitary somatotrophs and gonadotrophs likely reflect physiological changes in hormone packaging or secretion that have not been described previously. This study additionally produced numerous monoclonal antibodies which recognized PRL in chicken and/or turkey pituitary sections, including PR1G2B6 described here.

Our results demonstrate a striking change in the co-localization of CgA with GH and LH in chickens and turkeys of different ages. In young birds, many gonadotrophs that contained LH also contained CgA. Often, the CgA immunoreactivity was localized within discrete areas of the cell, suggesting compartmentalization of secretory granules containing CgA. In adults, dual-labeled gonadotrophs were extremely rare. We cannot determine from the present results whether these changes in gonadotroph content of CgA protein may be related to age or reproductive state. The mammalian CgA gene is known to have an estrogen response element (Iacangelo et al., 1991; Wu et al., 1991), and estradiol has been shown to specifically suppress CgA expression and dense-core secretory granule formation in rat gonadotrophs (Watanabe et al., 1998a). In the adult rat, gonadotrophs contain both CgA and CgC (secretogranin II), and in males FSH is packaged with CgA while LH is associated with small granules containing CgC (Watanabe et al., 1998b). Watanabe et al. (1991, 1998a) have suggested that CgA and CgC may mark two distinct regulated secretory pathways and function in the differential regulation of LH and FSH secretion. However, granins are unlikely to serve this function in the chicken, as LH and FSH are localized in separate gonadotrophs from early embryonic development through the adult (Proudman et al., 1999; Puebla-Osorio et al., 2002). Further studies are needed to determine whether CgA immunoreactivity is specifically suppressed in avian gonadotrophs by estradiol. The chicken may serve as a good model for separately studying the role of CgA in regulating LH and FSH secretion.

In contrast, chicken and turkey somatotrophs were devoid of CgA immunoreactivity in the young bird but were frequently dual-labeled in adult laying hens. Chromogranin A does not seem to be present in somatotrophs in mammals. In the rat, CgA is restricted to gonadotrophs (Watanabe et al., 1991, 1998a). Bassetti et al. (1990) co-localized CgA in multi-hormonal mammosomatotrophs in the bovine pituitary, but the CgA was not contained in GH-immunoreactive granules, nor was it present in somatotrophs containing only GH. If CgA marks a distinct regulated secretory pathway, as suggested for gonadotrophs, then the presence of CgA in some, but clearly not all, adult avian somatotrophs may imply heterogeneity in somatotroph regulation or function. Kim et al. (2001) have shown that absence of CgA may switch hormone secretion from the regulated to the constitutive pathway, so the presence of CgA in some cells may reflect a regulated secretory pathway that is absent in some somatotrophs. However, it is unlikely that the absence of CgA in somatotrophs of the young bird marks constitutive GH secretion, since secretion of this hormone is highly regulated in a pulsatile manner in the growing bird (Shaw et al., 1987). It is possible that this regulated secretion may occur through another granin protein, such as CgC.

We did not observe co-localization of CgA and PRL in either immature or laying birds. Chromogranin A may be unnecessary in lactotrophs, since PRL (unlike LH and GH) can self-aggregate (Watanabe et al., 1998b). However, it is also possible that lactotrophs may contain different granins (Lloyd et al., 1992; Ozawa et al., 1994).

Avian CgA has previously been identified in ostrich (Lazure et al., 1990) and chicken (Kameda et al., 1998). Lazure et al. (1990) isolated and sequenced a 76-amino acid peptide homologous to the N-terminal CgA peptide of mammals, and also sequenced a small peptide fragment homologous to the carboxy-terminal region of CgA. The de novo sequenced turkey CgA tryptic peptides were 95% identical to the chicken CgA sequence (regarding Leu/Ile differences as non-homologous), and our peptides 6 and 7 were 88% identical to the corresponding ostrich sequences (Lazure et al., 1990). Chicken pituitary CgA has been co-localized with LH in both the pars tuberalis of the pituitary gland, and in the cephalic lobe of the anterior pituitary in the embryo and young male chicken (Kameda et al., 1998).

In summary, the present study shows, for the first time, age-related changes in CgA content of gonadotrophs and somatotrophs, which may reflect a role for this granin in modulation of hormone secretion.

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